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Role of *Acinetobacter* for Biodegradability of Quaternary Ammonium Compounds

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Quaternary ammonium compounds (QACs) are cationic microbicidal substances which are important ingredients of disinfectants used for the disinfection of surfaces and medical instruments (Russel *et al. 1992).* The use of QACs as microbicidal ingredients increased during the last decade as substitute for aldehydes due to the health risk of aldehydes to humans. QACs are emitted via hospital effluents into municipal sewage (Kümmerer *et al. 1997)* and can disarrange biological waste water treatment. The prevalence of QACs resistant bacteria is elevated in effluents of sewage treatment plants receiving sewage containing QACs (Hingst *et al.* 1995).

QACs are reported to be not readily biodegradable (De Wart 1986, Van Ginkel 1991, ECETOC 1993). Because of the adsorption of QACs contradictory results of biodegradability, elimination and toxic effects of these substances against microorganisms in different biodegradability tests were reported (Gerike *et al.* 1978, ECETOC 1993, Sanchez Leal *et al.* 1994). One reason for the diverging test results may be that up to now possible interactions between QACs and inoculum i.e. toxicity against the bacteria used and adaptation were not investigated. But such a closer look is helpful for a better understanding of the test results and the evaluation of possible resistance of some bacteria to the tested biocides, as QACs have only little effect against gram-negative bacteria (Russel *et al. 1997).* For example, it is well known that bacteria may adapt to xenobiotic compounds (Aelion *et al.* 1988). In this study the biodegradability of two surface disinfectants A and B containing QACs (benzalkonium chloride and dimethyldidecylammonium chloride, ,,DDMAC") as main microbicidal compounds was investigated.

The Closed Bottle Test (CBT) is a test with low bacteria density and low organic carbon concentration simulating biodegradability in surface water. The colony forming units (CFUs) were determined in all test mixtures to monitor the toxicity against cultivable environmental bacteria in the test mixtures. Additionally, bacteria were cultivated and classified from a test mixture containing disinfectant B using the API®- and Biolog® Systems and ARDRA (amplified ribosomal DNA-restriction analysis). The biodegradability of QAC and the shift of gram-negative bacterial

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populations caused by this compounds is subject of discussion.

MATERIALS AND METHODS

The two disinfectants A and B as well as benzalkonium chloride kindly provided by Bode Chemie GmbH (Hamburg, Germany) were tested. Disinfectant A had the following composition: 3% benzalkonium chloride, 3 % DDMAC, 5% glutaraldehyde and 1-10% anionic surfactant of the alcohol ethoxylate type. The second disinfectant (B) had the following composition: 20% benzalkonium chloride, 5% alkyltriamin, 20-30% glycerin and 1-10% anionic tensides (alcoholethoxylate type). The test concentration of each disinfectant in the CBT was 2 mg/l corresponding to 0.06 mg/l and 0.4 mg/l benzalkonium chloride, for disinfectant A and B, respectively, and 0.06 mg/l DDMAC for disinfectant A, i.e. a total of 0.12 mg/l QAC. The growth inhibition test was performed to ensure that no inhibition in the CBT will occur at test concentration. The growth inhibition test with *Pseudomonas putida* was performed according to international standard method (IS0 1995). Concentrations were doubled eight times beginning with 1 mg/l. The following modification was made as described elsewhere in detail (Kümmerer *et al.* 1996, Al-Ahmad *et al. 1997):* The disinfectant benzalkonium chloride was used as control for 100% inhibition at a concentration of 32 mg/l. Incubation temperature was 30°C for better growth. The nutrition solution was adapted for waste water bacteria (Brözel and Cloete 1992, Kümmerer *et al.* 1996). The growth of bacteria was monitored by measuring the protein content according to Lowry *et al.* (1951). The CBT was conducted according to test guidelines (OECD 301 D) in the dark at 20 ± 1°C (OECD 1992) as described elsewhere in detail (Kümmerer *et al.* 1996, Al-Ahmad *et al.* 1997). Oxygen concentration was determined with an oxygen electrode (Oxi 196 with EO 196-1.5, WTW Weilheim, FRG) according to international standard method. Inoculum was sampled from the effluent of a municipal sewage treatment plant (Grezhausen, Landkreis Breisgau-Hochschwarzwald, Germany, population equivalent 70,000) the day before the beginning of the test. Sodium acetate of analytical grade was used as reference compound. In addition to the test guidelines, the CFUs were determined using a spiral plater (Lähden, Germany) and the agar medium for better toxicity control described elsewhere (Kümmerer *et al.* 1996). Because of the activity of QACs against bacteria, isolates from the test compound mixtures with product B containing highest QAC concentration were classified to investigate the adaptation of the bacteria to the test compound. 31 bacteria were isolated using a spiral plater (Lahden, Germany) on the 12th day after the beginning of the CBT from the test mixture containing the disinfectant. The agar medium used was adapted to waste water bacteria by adding pyruvate to the standard medium as described in detail elsewhere (Kümmerer *et al.* 1996).

After gram staining and aminopeptidase test the isolated gram-negative

bacteria were classified by using the API 20 NE test (No. 20050, bio Mèrieu, Mercy è Eloile, France). The Biolog Test System (Biolog Cooperation, Hayward, USA) for gram-negative bacteria was also used (Klingler *et al.* 1992).

Additionally, a restriction analysis after amplification of the 16s rRNA genes was conducted as described by Vaneechoutte *et al.* (1992). For the amplification the primers RI n (5'GCTCAGTTGAACGCTGGCG3') and B-U2 (5'ACATTTCAACACGAGCTG3') were used. Both primers were custom-synthesized by Pharmacia Biotech (Freiburg, Germany). 25 µl reaction mixtures were prepared with 1.25 U Taq polymerase (Pharmacia Biotech, Freiburg, Germany), 1x PCR buffer (Pharmacia Biotech), 0.2 mM (each) dinucleotide triphosphate (Pharmacia Biotech), 0.2 pmol (each) primer, and 1 µl template DNA. To prepare the template DNA, one bacterial colony was resuspended in 50 ul lysis buffer (1% tween 20/1x PCR buffer). The suspension was heated for 5 min at 95°C. The cell debris was sedimented at 16.000xg for 5 min in a microcentrifuge. An aliquot of the supernatant was used as a source of template DNA. The reaction mixtures were cycled in a thermocycler (Landgraf Varius V 45, Landgraf Laborgeräte, Langenhagen, Germany) as follows. The temperature program for the amplification was 120 s at 94°C; 30 cycles of 40 s at 94°C, 60 s at 50°C, 60 s at 72°C, and 180 s at 72°C. The amplification products were separated by- horizontal electrophoresis in 1% agarose and visualized by ethidium bromide staining. The amplification products were restricted with *Hinf I* (Pharmacia, Biotech) and *Hha* (Pharmacia, Biotech) and separated by horizontal gelelectrophoresis (in 5% acrylamide -7 M urea - 1x Tris - borate - EDTA gels) followed by a silver staining (Bassam *et al.* 1991). The type strains *A. baumannii* CIP 70.34 (ATCC 19606^T) was obtained from the Collection de I ' lnstitut Pasteur, Paris, France. *A. calcoaceticus* LMD 22.16 (ATCC 23055^T), *A. lwoffii* LMD 83.25 (ATCC 15309^T), and *A. junii* LMD 58.1 were obtained from the Type Culture Collection, Kluyver Laboratory of Biotechnology, University of Technology, Delft, The Netherlands. The *Acinetobacter* strains were grown at 20°C in FPA medium at a pH of 7.0 as described in detail elsewhere (Wiedmann-Al-Ahmad *et al.* 1994).

RESULTS AND DISCUSSION

Both products A and B exhibited no growth inhibition of gram-negative *Ps. putida* up to a concentration of 8 mg/l. 100% inhibition was observed at a concentration of 16 mg/l for both products. Benzalkonium chloride caused an inhibition of 100% at a concentration of 4 mg/l (Tab. 1). In the CBT product A was biodegraded up to 53%, whereas product B was biodegraded only up to 20% (Tab. 1). Since the difference between the measured biodegradation of the toxicity control mixture and the expected biodegradation computed from the test results of product A and B,

Table 1. Results of growth inhibition test with *Pseudomonas putida* and the biodegradability and toxicity in the Closed Bottle Test (CBT)

test compound	growth inhibition test IC_0 in mg/l	growth inhibition test IC_{100} in mg/l	CBT biodegradability in %	toxicity in CBT	
				toxicity control	CFU monitoring
Product A	8	16	53	-	-
Product B	8	16	24	-	-

-: no toxicity

respectively with the pure substances never exceeded 25%, no toxicity needs to be stated. The colony forming units (CFUs) were one order of magnitude lower in the blank than in the other test vessels. The CFUs were in the same range in the other test vessels indicating no toxicity of the products against the inoculum (Fig. 1). All isolated bacteria were gram-negative as evidenced by gram-staining and amidopeptidase test (Bactident®, Merck, Germany). With the exception of two *Pseudomonas* isolates, only members of the genus *Acinetobacter* were identified with the Biolog Test System as well as with the API Test System. The patterns of the restriction analysis confirmed these results. Two patterns were shown which belong to the genus *Pseudomonas* and *Acinetobacter,* respectively. It is well known that QACs are inefficient against many gram-negative bacteria (Russel *et al.* 1992). Resistance of Staphylococci and *Pseudomonas* against QACs is described in the literature (Jones *et al.* 1989, Heir *et al.* 1995, Russel *et al.* 1997). As all isolates from the test mixture with product B (20% benzalkonium chloride) belonged to the genus *Acinetobacter* with the exception of *two Pseudomonas* isolates as shown by ARDRA, we assume an important role of *Acinetobacter* for the biodegradation of QACs as well as for the resistance against these compounds. Because of the inadequacy of culture-dependent methods for isolating and classifying bacteria populations of complex structures (Wagner *et al.* 1993), the role of *Acinetobacter* cannot be described sufficiently only by relying on the above results. Furthermore, biodegradability of benzalkonium chloride in a prolonged CBT was higher than 80% only after 60 days (Steger-Hartmann 1995). This indicates a slow adaptation of bacteria in the CBT. As the concentration of the disinfectants (2 mg/l) was only 25% of the non-inhibitory concentration (IC_0 ≥8 mg/l) in the *Pseudomonas putida* growth inhibitory test, no growth inhibition of the bacteria in the test mixtures was expected, indicating no inhibition of the inoculum in the test vessel (Nyholm 1991). This was confirmed by the results of the toxicity controls.

Monitoring the colony forming units was shown to be useful to interpret CBT results of some microbial active cytotoxics (Al-Ahmad *et al.* 1997, Kümmerer and Al-Ahmad 1997), because the CBT is conducted with low

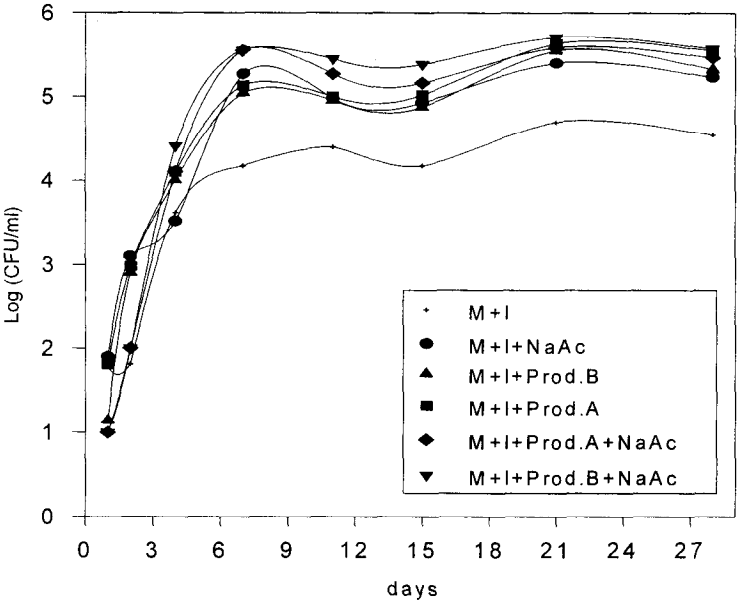

Figure 1. Colony forming units in Closed Bottle Tests with surface disinfectants: ■ Product A (M+I+Prod. A), ▲ Product B (M+I+Prod. B), ◆ toxicity control of Product A (M+I+Prod. A+NaAc), ▼ toxicity control of Product B (M+I+Prod. B+NaAc), ● quality control (M+I+NaAc) and ✚ blank (M+I).

bacterial density. The colony forming units (CFUs) also indicated no inhibition in the test mixtures. The determination of CFUs in the test vessels would indicate toxicity against some of the bacteria present, whereas according to the test guidelines toxicity against bacteria which could degrade benzalkonium chloride by oxygen consumption was detected. According to earlier results of chemotaxonomic investigation (Kümmerer and Al-Ahmad 1999), members of the genus *Acinetobacter* were responsible for the biodegradation of benzalkonium chloride. The results presented in this paper indicate that the elimination of benzalkonium chloride may be caused by biodegradation via gram-negative bacteria after some adaptation which probably occurs in sewage treatment plants (Hingst *et al.* 1995).

Furthermore, *Acinetobacter* may play an important role in developing resistance against QAC. Because of a possible selection of *Acinetobacter* in the growth agar taken, in further studies an in situ hybridization of the fixed inoculum with oligonucleotide probe for *Acinetobacter* should be used in the combination of classical microbiological methods to gain better insights into the behavior of quaternary ammonium compounds in surface water.

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